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Succinctus

The merozoite-specific protein, TgGRA11B, identified as a component of the *Toxoplasma gondii* parasitophorous vacuole in a tachyzoite expression model

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Abstract

The apicomplexan, *Toxoplasma gondi,* infects all warm-blooded animals as intermediate hosts but only felids as definitive hosts. Dense granule proteins are critical for the survival of *Toxoplasma* within host cells but, whilst these proteins have been studied intensively in tachyzoites, little is known about their expression in the coccidian stages in the cat intestine. Transcriptome profiling indicates that two putative dense granule proteins, TgGRA11A and TgGRA11B, are expressed uniquely in merozoites. Immunofluorescent microscopy of *Toxoplasma*-infected cat intestine and tachyzoites engineered to express TgGRA11B, reveals that it is a dense granule protein that traffics into the parasitophorous vacuole and its membrane.

Keywords

Toxoplasma gondii, Coccidia, merozoite, TgGRA11B, dense granule, parasitophorous vacuole

Toxoplasma gondii is an apicomplexan parasite with a unique ability to infect, seemingly, any warm-blooded animal as an intermediate host but with felids as the only definitive host. It consequently has a very complex life cycle. In keeping with this complexity, *T. gondii* possesses four invasive forms – sporozoites, bradyzoites, tachyzoites and merozoites – the former two being involved in transmission from host to host and the latter two being critical for rapid population expansion by asexual reproduction in the intermediate and definitive hosts, respectively (Ferguson, 2004). All four of these forms of the parasite have the ability to attach to, invade and modulate host cells, mobilising proteins on their surface and within intracellular organelles such as micronemes, rhoptries and dense granules in an exquisitely well-coordinated sequence that results in the formation of a membrane-bound parasitophorous vacuole around the parasites, within the cytoplasm of the host cell (see Carruthers and Boothroyd, 2007; Mercier and Cesbron-Delauw, 2015 for reviews).

Dense granule proteins (GRAs) are pivotal to the maturation and maintenance of the parasitophorous vacuole. Some GRAs are involved in the formation of an intravacuolar network, others in mediating uptake of nutrients from the host and a third group is exported across the parasitophorous vacuole membrane into the host cytoplasm and nucleus, modulating the host cell cycle and immune response (reviewed by Mercier and Cesbron-Delauw, 2015). Thus far, forty GRAs have been identified in *T. gondii*, mostly in tachyzoites (Nadipuram et al., 2016), though many are also found in sporozoites and bradyzoites (Fritz et al., 2012a, 2012b; Behnke et al., 2014). Although dense granules remain a conserved feature in merozoites, the invasive and rapidly replicating stages of the coccidian life cycle in the feline intestine, there is very little overlap between the expression profiles of individual GRAs in this stage compared with the other rapidly replicating form of *T. gondii,* the tachyzoite (Behnke et al., 2014; Hehl et al., 2015); indeed, until recently, only two GRAs have been found to be definitely expressed in merozoites (NTPase 1 and GRA7) and both of these are expressed across different stages of the life cycle (Ferguson et al., 1999a, 1999b; Ferguson, 2004). However, our transcriptome profiling

48 revealed that the *gra* genes with the highest transcript levels in merozoites of *T. gondii*, *gra11a*
49 (TGME49_212410) and *gra11b* (TGME49_237800), are siginificantly dowregulated in the tachyzoite
50 stage (Hehl et al., 2015); the definitive localisation of these unique GRAs has not yet been addressed.

51 TgGRA11A and TgGRA11B share 66% identity at the amino-acid level, with both predicted to
52 comprise a signal peptide and three transmembrane domains (Fig. 1a - TgGRA11B). However, our
53 homology searching failed to uncover significant homology to any other previously characterised
54 protein or protein domain. Therefore, in order to investigate a potential role for TgGRA11B in
55 merozoite biology, localisation studies were carried out using polyclonal mouse sera raised against a
56 recombinant protein that corresponds to the more divergent region of TgGRA11B (Fig. 1a). The
57 recombinant TgGRA11B protein was expressed in bacteria using the pET41a(+) system (Novagen®)
58 and purified under denaturing conditions using NiNTA agarose (QIAGEN) as per manufacturer's
59 instructions, and polyclonal mouse sera was produced subsequently, as described previously (Walker et
60 al., 2015). Immunofluorescent microscopy, using this antisera, showed staining consistent with the
61 localisation of TgGRA11B within dense granules of intracellular merozoites in feline enterocytes (Fig.
62 1 b-e) at early (days 3 and 5 p.i.) stages of asexual development. Later in infection (day 7 p.i.), staining
63 of infected enterocytes in vivo indicates that TgGRA11B traffics, first, to the parasitophorous vacuole
64 and, subsequently, to the parasitophorous vacuole membrane (Fig. 1 f-g). Unfortunately, however,
65 definitive assignment of localisation of TgGRA11B to merozoite dense granules and parasitophorous
66 vacuoles in vivo is currently not possible because of a lack of verified specific markers for these
67 structures in the coccidian stages of development of *T. gondii.* Therefore, we engineered a tachyzoite
68 expression model for TgGRA11B (Fig. 2).

69 Construction of the pTubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-sag1-Lox (Fig. 2a)
70 plasmid was carried out as follows: first, the 711 bp coding sequence of mCherry and adjacent 3'
71 untranslated region (UTR) of dihydrofolate reductase-thymidylate synthase (*dhfr*) was PCR amplified

from the pmCherry.LIC.DHFR plasmid (Huynh and Carruthers, 2009) using primers ***ATGCAT***ATGGTGAGCAAGGGCGAG (***nsii***) and TGTCACTGTAGCCTGCCAGA and cloned into pGEM®-T Easy (Promega) via AT-cloning; second, plasmid sequencing was carried out to select clones where the pGEM®-T Easy plasmid *nsii* site was positioned 62 bp from the insert *nsii* site; next, the minimal 493 bp alpha tubulin (*tuba* or *tgme49_316400*) promoter and adjacent start codon were amplified from CZ *T. gondii* genomic DNA using primers ***ATGCAT***GGATCCATCGTCTAGAGTCCCGCGTTCGTGAA (***nsii***) and ***ATGCAT***TTTGTCGAAAAAGG (***nsii***) and cloned into pmCHERRY-dhfr via *nsii*; third, the 84 bp coding sequence for a triple haemaglutinin tag (HA3) was PCR amplified from pHA3.LIC.DHFR (Huynh and Carruthers, 2009) using primers ***CATATG***<u>*GATATC*</u>TACCCGTACGACGTCCCG (***ndei***, <u>*ecorv*</u>) and ***CATATG***GGCATAATCTGGAACATCGTAAG (***ndei***) and cloned into pTubA-mCHERRY-dhfr via *ndei*; fourth, the entire 1,413 bp coding sequence of *tggra11b* (*TGME49_237800*), excluding the stop codon, was PCR amplified from CZ *T. gondii* genomic DNA using primers ATGTCCCACCGCATGGCAT and TGGCTTCAACTCGTCCTCTTCC and inserted in-frame with the HA3-mCHERRY coding sequence by blunt-end cloning; a Lox-flanked dhfr-DHFR-sag1 plasmid was created in parallel by PCR amplifying the pyrimethamine-resistant DHFR with its own promoter from pHA3.LIC.DHFR (Huynh and Carruthers, 2009) using primers ***ATCGAT***AAGCTTCGCCAGG (***clai***) and ***TTAATTAA***ACAGCCATCTCCATCTGGAT (***paci***) and cloning into pLox-TubA-CAT-sag1-Lox (Brecht et al., 1999) via *clai* and *paci* (i.e. replacing the TubA-CAT sequence); and, finally, the Lox-flanked pyrimethamine-resistant *dhfr* cassette was inserted adjacent to the TubA-TgGRA11B-HA3-mCHERRY-dhfr cassette via *noti* and *apai*.

The entire pTubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-sag1-Lox reporter cassette was PCR amplified using vector-specific M13R and M13F primers and co-transfected as donor DNA into CZ *T. gondii* tachyzoites with the pCas9:sgUPRT plasmid and selected with 1 µM pyrimethamine, as

described previously (Shen et al., 2014). CRISPR/Cas9 mediated integration of the construct at the *tguprt* genomic locus (exon 5) was confirmed for individual pyrimethamine-resistant clones by PCR screening using primers GCATGCAGCCATATACGAAA and GCCTATCGCTCTTCACCTTCT (PCR 1; 600 bp) and GCATGCAGCCATATACGAAA and ATGCATTTTGTCGAAAAAGG (PCR 2; 962 bp) (Fig. 2a). PCR analysis of the pyrimethamine-resistant clone 1 revealed the correct insertion of the reporter cassette into exon 5 of *tguprt* (Fig. 2b).

Antibodies to both HA and recTgGRA11B co-localise with putative dense granules (Fig. 2c) and the parasitophorous vacuole (Fig. 2d) of tachyzoites engineered to express TgGRA11B, when using permeabilisation with either 0.25% Triton X-100 or 0.01% saponin, respectively. Antibodies to HA also co-localise with the antibody to the bona fide dense granule protein after permeabilisation with Triton X-100, GRA1 (Fig. 2e). Furthermore, the same two antibodies label the parasitophorous vacuole of TgGRA11B-expressing tachyzoites under gentle permeabilisation conditions with saponin (Fig. 2f). Counterstaining with the tachyzoite surface protein, SAG1, clearly distinguishes between the parasite plasma membrane and the parasitophorous vacuole, which is labeled with anti-HA antibodies (Fig. 2g). Finally, immunofluorescent microscopy detected mCherry signal in putative dense granules and the maturing parasitophorous vacuole of tachyzoites expressing the merozoite-specific TgGRA11B-(Fig. 2h).

In conclusion, using immunofluorescent microscopy on sections of *Toxoplasma*-infected cat intestine and by using a novel model where tachyzoites are engineered to express the merozoite-specific TgGRA11B, we have been able to demonstrate that this protein is, indeed, a dense granule protein. Furthermore, we present evidence that it is trafficked from parasites into the maturing parasitophorous vacuole and, subsequently, into the parasitophorous vacuole membrane surrounding *T. gondii* within its host cell – in this case, uniquely, the enterocyte of the feline intestine. This is in keeping with the protein's possession of an N-terminal hydrophobic signal peptide and three hydrophobic α-helices,

features that, in numerous other GRAs, are associated with secretion into the parasitophorous vacuole and subsequent localisation in its membrane (Mercier and Cesbron-Delauw, 2015).

Ethics statement

Experiments involving animals were performed under the direct supervision of a veterinary specialist, and according to Swiss law and guidelines on Animal Welfare and the specific regulations of the Canton of Zurich. Permit number 108/2010 covers all animal experiments presented in this paper and was approved by the Veterinary Office and the Ethics Committee of the Canton of Zurich (Kantonales Veterinäramt Zürich, Zollstrasse 20, 8090 Zürich, Switzerland).

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Figure 1. In vivo localisation of native TgGRA11B in a *Toxoplasma gondii*-infected cat intestine. (a) Schematic representation of the predicted protein structure of TgGRA11B including the location of a signal peptide (SP) and three transmembrane domains (TM1, TM2, TM3). Polyclonal antisera were raised against recTgGRA11B, a bacterially expressed recombinant protein corresponding to a region of the predicted TgGRA11B protein structure that encompasses TM2 and TM3 (indicated by a horizontal black line). (b-g) Sections of paraffin-embedded *T. gondii*-infected cat intestine from different post-infection time-points with the CZ strain (Hehl et al., 2015) were assessed by immunofluorescent microscopy. At early stages of *T. gondii* asexual expansion at days 3 (b, c) and 5 (d) post-infection with tissue cysts of CZ *T. gondii* (as described by Hehl et al., 2015), merozoites (surface-stained in green with polyclonal antisera from a sheep naturally infected with *T. gondii*; Hehl et al., 2015) are characterised by TgGRA11B localisation (red) that is consistent with dense granule cargo. In later stages of merozoite development, at day 7 post-infection, TgGRA11B (green) is detected not only in these putative dense granules (e, f) but also in the parasitophorous vacuole and parasitophorous vacuole membrane (f, g). DAPI counterstain was used for nuclear staining (b-g). The antisera to recTgGRA11B did not label any other stages of development of *T. gondii.* Scale bars are 5μm.

Figure 2. Genetic engineering and immunolocalisation of TgGRA11B in a tachyzoite expression system. (a) Schematic representation of the *tubA-TgGRA11B-HA3-mCHERRY-dhfr:Lox-dhfr-DHFR-sag1-Lox* reporter construct used for site specific integration into the *TgUPRT* genomic locus in wild-type CZ *T. gondii* tachyzoites (WT). A clonally isolated mutant (clone 1) is also schematically represented, demonstrating site-specific insertion of the reporter construct. (b) Diagnostic PCR demonstrated correct insertion of the reporter construct in clone 1 (with a positive product for the PCR 2 primer set; negative for PCR 1) compared with the untransfected wild-type (WT) parental line (PCR 1 positive; PCR 2 negative). (c-h) Immunolocalisation was carried out on in vitro tachyzoite stages of the *TgGRA11B-HA3-mCHERRY* mutant (clone 1) at 24h post-inoculation. The exogenous TgGRA11B-HA3-mCherry was localised using rabbit anti-HA antibody (red; Stewart et al., 2016) and the mouse anti-recTgGRA11B antibody (green) in putative dense granules of tachyzoites using 0.25% Triton X-100 permeabilisation (c) and in the parasitophorous vacuole using a gentler 0.01% saponin permeabilisation (d). TgGRA11B-HA3-mCherry (red) co-localised with antisera to a bona fide dense granule protein, TgGRA1 (green; Stewart et al., 2016), in dense granules (0.25% Triton X-100, e) and in the parasitophorous vacuole (0.01% saponin, f). (g) Counterstaining with mouse anti-TgSAG1 antibody (green; Stewart et al., 2016), distinguished the parasitophorous vacuole-staining of TgGRA11B-HA3-mCherry (red) from the tachyzoite surface (green; 0.01% saponin). (h) Similarly, native mCherry fluorescence from TgGRA11B-HA3-mCherry also confirmed dense granule localisation in early stages of tachyzoite expansion (first image), with parasitophorous vacuole localisation in later development (second and third images). Anti-HA and anti-TgGRA11B antibodies failed to stain tachyzoites of the wild-type parental strain and, likewise, no mCherry signal was detected in wild-type parental tachyzoites (data not shown). Scale bars are 2μm.